Bacterial Species Associated with Wetwood of Elm

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ABSTRACT

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To investigate the etiology of bacterial wetwood in elm (*Ulmus americana*), we isolated microflora from wetwood capillary liquid, wetwood, and unaffected sapwood of branches, trunks, and roots. Stem and branch isolation samples were obtained from 52 elm trees, 35–85 cm in diameter at 1.3 m above mean ground level, and from the roots of 10 trees 42–78 cm in diameter at 1.3 m stem height. Enrichment broth culturing for 48–96 hr under aerobic and anaerobic conditions, followed by repeated streaking on agar plates, was used to obtain pure cultures. Fourteen species of bacteria and two species of yeasts were isolated. *Enterobacter* and *Klebsiella* species were most often isolated from wetwood tissues. They occurred in greater numbers than *Pseudomonas* and *Bacillus* species which were most frequent in unaffected sapwood. The data show that an association of bacterial species produce the complex of symptoms observed.

Bacterial wetwood of American elm is characterized by an association of water-soaked tissue, high pH levels, and a bacterial flora. The wetwood condition is prevalent in all large elms examined, is believed to be present in all mature elms, and occurs in varying degrees in other tree species (6,7,21). Wetwood occurs in the central core of senescent xylem of large stems, where it is considered nonpathological or in younger, responsive outer sapwood, where it is often pathological. Nonpathological wetwood is characterized by a central core of brown to black, fetid-smelling, water-soaked tissue surrounded by numerous, healthy, nondiscolored, annual sapwood rings in intact stems. In contrast, pathological wetwood is characterized by liquid exuding from wounds of external origin, eg, branch stubs or bark cracks, vertical streaks of white to gray encrustations of dried effluent on bark surfaces, and tan to brown liquid-soaked bark infested with an external microflora (wetwood slime). The exuding liquid ("bleeding" sap or wetwood capillary liquid) is forced from infected xylem through wounds by gas pressures caused by bacterial fermentation in the wetwood (2,8,10,24).

Bacterial wetwood in elm was studied intensively by Carter (2). He named a new species of bacteria (*Erwinia nimipressuralis* Carter) as the causal agent of wetwood, and established "exterior" or "bleeding" wetwood as a disease condition (2). However, the validity of *E. nimipressuralis* as a primary pathogen has not been established (5). Recent evidence on bacterial wetwood in elm, willow, poplar, and maple indicate that two or more bacterial species may be involved simultaneously in the development of wetwood (6,7,9,11,17,20–22). New evidence indicates that *E. nimipressuralis* was misnamed, and is now classified as an *Enterobacter* sp. biochemically similar to *E. cloacae* (1,5).

Since Carter's initial work (2), bacteria associated with elm wetwood have been studied by others (9,15–17,24). The purpose of this study was to determine the microflora associated with elm sapwood, wetwood, and wetwood capillary liquid in Maine.

MATERIALS AND METHODS

Isolation of bacteria. Isolations were attempted from 1,400 samples of wetwood capillary liquid, wetwood, or unaffected sapwood from trunks and branches of 52 trees, and from roots of 10 other trees. The trees (*Ulmus americana* L.) ranged in diameter from 35–85 cm at 1.3 m above mean ground level and were located within 5 km of the University of Maine campus at Orono. Sample trees were selected from planted stock along roads. Sampling was done during May through September for a 3-yr period.

Wetwood capillary liquid was extracted from holes made through unaffected, nondiscolored outer sapwood into darkly colored wetwood tissue at 1.0 m stem height. Bark and phloem were removed with a 2.5-cm-diameter machinist's punch. The exposed wood was surface sterilized with 70% ethanol. Holes were made in the xylem with a sterile increment borer. After removal of the borer and wood core, a sterile, plastic nipple was inserted tightly into the hole, and rubber tubing was attached to a sterilized collection flask into which capillary liquid was exuded by positive pressure within the wetwood.

To obtain sapwood and wetwood samples, bark was removed at 1.0 m stem height, the wood surface was sterilized with 70% ethanol, and a sterile increment borer was used to extract cores from nondiscolored, unaffected sapwood or discolored wetwood in stems, branches, and roots. Care was taken not to sample unaffected sapwood closer than 1 cm from wetwood.

Five 1-ml aliquots of stem wetwood capillary liquid and five increment core lengths (2 cm) of sapwood and nonpathogenic wetwood from each position sampled in each tree were placed immediately in tubes containing 10 ml of trypticase soy broth without dextrose (TSB), reduced thioglycollate broth, nutrient broth (NB), and potato-dextrose broth (PDB) to initiate enrichment cultures. At least one additional control sample of wetwood capillary liquid, and sapwood and wetwood from each location in each tree was autoclaved at 121 C, 10.5 kg/cm^2 for 20 min before being placed in a duplicate series of broth tubes to test accuracy of isolation methods. Two broth cultures per position per medium were incubated aerobically at 23 C, two were incubated aerobically at 37 C, and the remaining culture was incubated anaerobically in a anaerobe chamber under a gas mixture of 5% H_2 and 10% CO_2 at 36.5 C. After 48–96 hr, loopsful of the broth cultures were streaked in triplicate on plates of trypticase soy agar without dextrose (TSA), trypticase soy agar with 5% sheep's blood added (TSAw), nutrient agar (NA), and potato-dextrose agar (PDA) and incubated under conditions previously stated.

Identification of bacteria. Pure cultures of isolated bacteria were identified using standard bacteriological methods (18) at 23 and 37 C. API (Analytab Products #6010-25, Plainview, NY 11803) identification strips incubated at 37 C for 24–48 hr were used as reference standards.

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RESULTS

Occurrence of bacteria in elm wood and capillary liquid. Five hundred sixty-eight bacterial strains were obtained during the 3-yr study period. From these, 82 representative strains were purified and identified (Table 1). The five most common bacterial strains from wetwood and wetwood capillary liquid are shown in Table 2. There were differences between wetwood associated bacterial isolates in stems, branches, roots, and wetwood capillary liquid. Bacteria were isolated infrequently from unaffected sapwood, where only 7.0% of the samples yielded bacterial isolates. Aerobic pseudomonads and *Bacillus megaterium* were the most common isolates from unaffected sapwood, present in 7.0 and 3.0% of the samples, respectively.

Of all bacterial isolates, 71% were obtained with the combined use of TSB and TSA or TSAw. No mycelial fungi were isolated from wetwood capillary liquid, wetwood or unaffected sapwood in any of the samples.

Taxonomy of fermentative, Gram-negative bacterial isolates. Characteristics of the Gram-negative, fermentative bacteria isolated from wetwood and wetwood capillary liquid are summarized in Table 3. All strains fermented glucose in the Hugh-Leifson oxidative versus fermentative test, did not produce H_2S, and were oxidase-negative and catalase-positive. *Enterobacter agglomerans* and *Klebsiella oxytoca* strains were biochemically similar to previously described strains. *Enterobacter cloacae* (metabolically atypical) exhibited aberrent reactions in the production of acetylmethyl carbinol and in the fermentation of some sugars. Other isolates identified as *Enterobacter* spp. were unlike previously described species. No attempt was made to assign a species name to these strains pending further investigation. These strains may be metabolically atypical variants of previously described species adapted to the wetwood environment.

DISCUSSION

Our observations do not support the conclusion of Carter (2) that bacterial wetwood in elm is caused exclusively by one bacterial species. A more likely hypothesis is that an association of bacteria and possibly yeasts, act in concert to produce the complex of symptoms observed.

Observations of diverse populations of bacteria in wetwood have been made in poplar (15), elm, cottonwood, and willow (16,24). These authors assumed that since most bacteria isolated are common soil and water inhabitants, infection could have occurred through root wounds. However, in elm, initial bacterial infection appears to occur in young elm stems less than 10 years of age (9). Bacterial infection of root tissue in elm was not seen in these early stages of wetwood development. The initial source of bacterial inocula is still assumed to be the soil, and root infections through wounds are considered secondary, occurring primarily in roots of older stems (9). Obligately anaerobic bacteria, especially methanogenic species, are very sensitive to free oxygen (23). The presence of large numbers of these bacteria in tree stems has been noted, but the origin of infection is not clear (10,14,21,22). It is assumed that initial infection must occur under conditions of strict anaerobiosis. Such conditions could occur in the soil and presumably in water-soaked elm tissue. Further studies are necessary to determine if methane is produced within the wetwood of young trees, or if it is only produced after secondary root infections have occurred in older trees.

Several types of culture media were used to obtain all possible bacterial and fungal isolates. For isolating wood-inhabiting bacteria, enrichment broth cultures are preferred over standard wood chip isolation techniques (3). Immersion of wood in liquid facilitates the free movement of motile (active) and nonmotile (passive) bacteria from the sample into the culture medium. In our study, trypticase soy-based medium was found to be the most useful.

The bacterium isolated most frequently from wetwood in elm by Carter (2) was described as a new species, *Erwinia nimipressuralis*. According to Dye (5), this species was biochemically similar to *Enterobacter cloacae* and its pathogenicity to living elm cells doubtful. In our study, we isolated *E. cloacae* from wetwood, but it was not the most frequent isolate. A possible explanation for this is that Carter used trees from the Midwest (IL), and we used trees from the Northeast (ME). If the major source of bacterial inocula is the soil, then we would expect a different bacterial flora in the cooler northeastern USA than the midwestern region. However, the results to date indicate that members of the Enterobacteriaceae may be the most common group in wetwood due to their ubiquitous nature and simple growth requirements which allow them to adapt to diverse ecological situations.

Most bacterial species isolated from elm wetwood, wetwood capillary liquid, or unaffected sapwood are normally classified as saprophytes (1), or in some cases as opportunistic human pathogens (9). The colonization of physiologically nonfunctional wood within a living tree stem by saprophytic bacteria is not de facto evidence of disease, because it does not cause physiological disturbance of the host unless the bacteria are released into

TABLE 1. Bacteria and yeasts isolated from wetwood capillary liquid, wetwood, and sapwood in American elm

Bacteria	Synonyms[a]	Origin[b]
Enterobacter cloacae	*Erwinia nimipressuralis*	CL,W-R,B,ST, S-R,ST
Enterobacter agglomerans	*Erwinia herbicola* *Erwinia lathyri* *Bacterium typhiflavum*	CL,W-R,B,ST, S-R,B,ST
Klebsiella oxytoca	*Klebsiella pneumoniae* (indole +)	CL,W-R,B,ST, S-ST
Serratia fonticola	*Citrobacter* spp.	CL,W-B,ST,S-ST
Bacillus megaterium		CL,W-ST,S-B,ST
Pseudomonas fluorescens		W-B,ST,S-R,B,ST
Pseudomonas spp. (3)[c]		CL,W-R,S-R,ST
Streptococcus mitis		CL,W-ST
Acinetobacter spp.		CL
Staphylococcus spp.		CL,W-ST
Unidentified obligately anaerobic bacteria (2)		CL,W-R,ST
Unidentified yeasts (2)		CL

[a] Older scientific names are shown for the sake of clarity.
[b] CL = stem wetwood capillary liquid, R = root, S = sapwood, B = branch, W = wetwood, and ST = stem.
[c] Number in parentheses indicates number of different species isolated.

TABLE 2. Occurrence of most common bacteria in American elm wetwood and wetwood capillary liquid

	Wetwood core		Wetwood capillary liquid[a]	
Bacterial species	Samples tested[b]	Percent	Samples tested[b]	Percent
Enterobacter spp.[c]	570	20	260	22
Enterobacter agglomerans	570	15	260	12
Klebsiella oxytoca	570	24	260	21
Bacillus megaterium	570	6	260	8
Pseudomonas spp.	570	18	260	22
Control (autoclaved)	52	0	52	0

[a] Samples taken from stem wetwood only.
[b] Samples were taken from 52 trees.
[c] Includes atypical *Enterobacter cloaceae* and all other *Enterobacter* spp. except *E. agglomerans*.

TABLE 3. Groups of fermentative, Gram-negative bacteria from wetwood and wetwood capillary liquid in 62 American elm trees

Group	Classification	No. of isolates	Positive reactions for indicated property[a]													
			I	M	VP	C	U	A	L	O	Gel	Lac	Mann	Sor	Suc	Ara	Rha
I	*Enterobacter agglomerans*	15	0	89	0	0	0	0	0	0	0	67	100	33	100	33	67
II	*Klebsiella oxytoca*	20	100	0	100	100	0	0	100	100	0	100	100	100	100	100	100
III	*Enterobacter cloacae*, metabolically atypical	12	0	67	42	100	0	100	0	100	25	58	92	50	50	100	100
VI	Other *Enterobacter* spp.	5	0	100	0	80	0	100	0	60	(−)	(−)	(−)	(−)	(−)	(−)	(−)

[a] Letter symbols and abbreviations: I = indole production; M = the methyl red test; VP = the Voges-Proskauer test; C = citrate utilization; U = urease production; A = arginine dihydrolase; L and O = lysine and ornithine decarboxylase; Gel = gelatine liquefaction; Lac, Mann, Sor, Suc, Ara, and Rha = (respectively) fermentation of lactose, mannitol, sorbitol, sucrose, arabinose, and rhamnose; and (−) = not tested.

responsive or living tissues.

A parallel can be drawn between the association of bacteria in elm wetwood and of those in the bovine rumen. Both systems utilize diverse populations of aerobic and anaerobic bacteria to degrade substrate material and produce methane as an end product (10,15). Aerobic and facultatively anaerobic bacteria at the sapwood-wetwood interface may reduce free oxygen content within the wood. This would lead to anaerobiosis and allow the microbial production of methane. One difference between fermentative processes in the bovine rumen and the elm tree is that cellulose is apparently not degraded in elm wetwood. No significant difference in most strength properties has been noted between sapwood and wetwood (7,15), although shearing strength is sometimes affected (21,22), and the wood condition known as "shake" has been reported to be associated with wetwood in elm and other hardwoods (2,20). In this study, known cellulose-degrading bacteria were not isolated. *Enterobacter*, *Klebsiella*, *Bacillus*, and *Pseudomonas* species isolated in this study did not degrade cellulose in the laboratory.

The presence of both aerobic and anaerobic bacteria, including two unidentified, obligately anaerobic species (Gram-negative rods), shows that free oxygen is present in certain areas of the wetwood, while absent in others. Microbial production of methane in wetwood of elm and other tree species indicates that conditions of strict anaerobiosis must occur in the stem wetwood (16,23,24). As reported for wetwood in black cottonwood, the absence of free oxygen within a tree stem could be advantageous to the host, precluding the growth of aerobic, wood-decaying basidiomycetes (19). Observations by the authors of over 1,000 trees indicated that wetwood in intact elm stems was rarely decayed, and the wetwood core of cut stumps was colonized by fungi much later than the surrounding ring of noninfected sapwood (9). The absence of fungi in wetwood is not surprising due to its alkaline pH (7.0–8.0), partial anaerobic character, and large populations of bacteria (7,14).

In our study, we concentrated on the isolation of aerobic and facultatively anaerobic bacterial species. Other investigators (15,16) have shown that significant numbers of obligate and facultative anaerobes are present in elm wetwood. In another study (13), population levels of total heterotrophic bacteria in elm wetwood capillary liquid were determined and found to be on the order of 10^6–10^7 cfu/ml. No correlation was found between different groups of bacteria in wetwood capillary liquid and soil from around stem bases (13). While we did not identify the two obligately anaerobic bacteria isolated in this study from elm wetwood, we did determine that they were not *Bacteroides* spp. These data are in agreement with Schink et al (15) who noted the presence of *Bacteroides* spp. in wetwood of cottonwood, but not in wetwood of elm. We also did not isolate any sporeforming anaerobic bacteria (*Clostridium* spp.) which have been shown to be present in elm wetwood in relatively high numbers (16). This difference could be explained by the effect of geographic variation on soil populations available for wetwood infection.

Obligately anaerobic bacterial populations in elm wetwood represent the end of a microbial succession associated with the phenomenon. Their presence in wetwood is at least partially dependent upon the action of aerobic and facultatively anaerobic bacterial species which remove O_2 from the wood, in effect favoring the development of an environment in which they can survive. The initial formation of wetwood in elm can therefore be assumed to be due to bacteria other than those shown to be obligate anaerobes. While the presence of strictly anaerobic bacterial populations in living trees is interesting, especially from the point of view of methane production and future uses of wood for hydrocarbon fuel (10), and while their presence does explain some of the wood degradation associated with wetwood in elm, their identification and study does little to explain how wetwood is initiated in living trees. The bacteria isolated in our study probably represent groups primarily involved with initial wetwood formation and development in elm and most are considered common soil and water inhabitants.

The presence of a wetwood core in every American elm examined indicates that bacterial wetwood may have no adverse effect on the host unless the host is wounded, and that the wetwood association may have evolved over a considerable period of time. Our data show that young elms are internally infected with mixed populations of bacteria and yeasts (9). If this early infection is confirmed, ensuing environmental and geographical factors could contribute to differences between the type and number of soil and water bacteria that are available to colonize wetwood in individual trees and populations of trees growing at different geographic locations.

Bacterial wetwood is, by definition, a disease only when it disturbs the normal physiology of the tree. But, within the central core of a healthy elm, wetwood is only a condition of the wood, in which the wood has a higher than normal moisture content associated with bacterial colonization. This distinction is important since wet pockets of wood also can form in the absence of microorganisms (4,12). Wetwood can be discolored (2,24) or nondiscolored (4). Terms such as pathological or false heartwood should be avoided because they infer that all wetwood conditions are associated with heartwood, while ignoring wetwood associated with wounds. In all bacterial wetwood diseases, wetwood is associated with dead or dying cells.

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